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Lack of midazolam-induced anxiolysis in the plus-maze Trial 2 is dependent on the length of Trial 1

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Abstract

The influence of the first exposure length upon the effect of midazolam (MDZ) administration prior to the second exposure in the elevated plus-maze (EPM) was investigated. Drug-free rats were assigned to freely explore the EPM for 1, 2 or 5 min (Trial 1). Twenty-four hours later, each group was subdivided in two further groups, which were retested in the EPM for 5 min, 30 min after either saline or MDZ (1.5 mg kg^{-1}) administration (Trial 2). The data showed that during Trial 2, the percentage of entries (%Open arm entries) and time spent in the open arms (%Open arm time) were decreased if rats were pre-exposed to the EPM for 2- or 5-min Trial 1, while the group submitted to 1-min Trial 1 length displayed decreased %Open arm time only. The anxiolytic effect of MDZ prior to Trial 2 was present in the group submitted to 1-min, impaired in the group submitted to 2-min and absent in the group submitted to 5-min Trial 1 length. Data are analyzed taking into account the emotional learning which underlies the exploratory behavior during the EPM Trial 2.

Keywords: Anxiety; Learning; Midazolam; Fear; Elevated plus-maze

1. Introduction

The elevated plus-maze (EPM; Handley and Mithani, 1984) is an animal model of anxiety based on the natural aversion of rodents for open spaces (Fernandes and File, 1996; Treit et al., 1993; Lister, 1987). The EPM is a task designed to detect anxioselective drug effects, but it is also a fear-eliciting task, per se, providing predictive and face validity as an animal model of anxiety (Rodgers and Cole, 1994). During the last 17 years, this model has provided increased knowledge about the neurobiology of anxiety in rodents and it has been widely used to screen new anxiolytic drugs (Hogg, 1996). A general aspect of the EPM exploration shows that rats avoid entering, and therefore spend less time exploring the open arms, with clear enclosed arms preference. This preference for the protected spaces has been ascribed as a sum of features including the rat's inability to engage in thigmotaxic behavior (Treit et al., 1993; Cardenas et al., 2001).

In recent years, the EPM task has incorporated some procedures and variables that increased its usefulness as a tool to study defensive behavior. One of the modifications was the incorporation of ethological measures, which increased the validity of this model, encompassing the effects of putative anxiolytic compounds with a mechanism of action different from that of benzodiazepines (BZ) (Cole and Rodgers, 1993; Cruz et al., 1994). The second modification was the inclusion of a retest session, which presupposes a learned component underlying the exploratory behavior during EPM re-exposure (File, 1990, 1993).

A remarkable and consistent finding related to the EPM is the lack of BZ-induced anxiolysis in rats with prior EPM experience, a phenomenon originally referred to as "one trial tolerance" (File, 1990). This phenomenon has been found to be independent of the drug state in Trial 1 and intertrial interval (File, 1993).

The hypotheses to explain the lack of BDZ-induced anxiolysis in the EPM Trial 2 include locomotor habituation (Dawson et al., 1994), sensitization of fear of the open arms (Rodgers and Shepherd, 1993) and a qualitative shift in the emotional state elicited by the subsequent exposure to the

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EPM (File and Zangrossi, 1993; Holmes and Rodgers, 1998), against which BDZs are ineffective (File, 1993; File and Zangrossi, 1993). On the other hand, several studies using rats have argued that "one trial tolerance," at least to the chlordiazepoxide effects, might be prevented by either lidocaine-reversible bilateral lesions of the basolateral amygdala immediately after Trial 1 (File et al., 1998) or the dorsomedial hypothalamus immediately before Trial 2 (File et al., 1999), as well as by increasing the length of EPM sessions (File and Zangrossi, 1993; Holmes and Rodgers, 1999). At the molecular level, it has been shown that [^{3}H]flunitrazepam but not [^{3}H]muscimol binding was increased in the amygdala and hippocampus immediately after 5 min of EPM exposure (Chacur et al., 1999), supporting the suggestion that the first maze experience would cause the release of endogenous compounds that bind to and alter the state of the BDZ receptor (Gonzalez and File, 1997).

Based on these findings, the following hypothesis may be formulated: if emotional learning is occurring throughout Trial 1, and from this learning a different type of fear emerges during Trial 2, which is insensitive to BDZ-like drugs, then it is possible that a reduction in the duration of Trial 1 would impair this shift towards a behavioral and neurochemical form of resistance to BDZ during Trial 2. The present study was designed to further evaluate the above question.

2. Method

2.1. Animals

Male Wistar rats weighing approximately 250 g were supplied by the animal house of the Federal University of Santa Catarina. The animals were housed in groups of five and underwent a period of adaptation for 7 days with free access to food and water, under a light/dark cycle of 12 h (lights on 6:00 a.m.). The animals were handled for weighing, drug administration and cleaning of the cages only. All the experimental procedures were conducted in compliance with recommendations of the "Principles of Animal Care" and of the "Ethical Principles of Animal Experimentation" of the Brazilian College of Animal Experimentation.

2.2. Apparatus

The EPM consisted of two opposed open arms (50 × 10 cm) and two other opposed arms of the same size, enclosed except for the entrance by wooden walls 40 cm high. In order to avoid falls, the open arms were surrounded only by a short (1 cm) Plexiglas edge. The four arms were arranged in such a way as to form a cross. The arms extended from a central platform (10 × 10 cm) and were raised 50 cm above the floor. Four 15-W fluorescent lights arranged as a cross 100 cm above the maze were used as the sole source of illumination. Each experimental session was recorded by a video camera in an adjacent room.

2.3. Drug

Midazolam (MDZ; Roche, Brazil) was dissolved in saline solution (0.9% w/v) and was administered by intraperitoneal route in a volume of 0.15 ml/100 g of body weight.

2.4. Procedure

Eighty-six drug-free rats were assigned to freely explore the EPM for 1 (n = 29), 2 (n = 25) or 5 min (n = 32); each animal was placed on the central platform of the maze facing an enclosed arm and was allowed to explore the maze for the appropriate time period (Trial 1). Twenty-four hours later, each group was subdivided in a further two (n varying between 8 and 17 rats), receiving either MDZ (1.5 mg kg^{-1}) or equivalent volume of saline; 30 min after drug administration, each animal was re-exposed to the EPM for 5 min (Trial 2). The standard spatio-temporal variables, such as the number of entries into either the open or enclosed arms, as well as the total number of arms entries, were recorded. The exploratory behavior upon the open arms was expressed as the mean percentage of entries into (%Open arm entries) and the time spent inside the arm (%Open arm time). Arm entry and arm exit were defined as all four paws into and out of an arm, respectively. In order to avoid odoriferous cues (Wallace et al., 2002) between animals, the maze was cleaned with wet (alcohol, 20% v/v) and dry cloths. Any animal which fell off the maze was excluded from the experiment. All the experiments were carried out during the light phase of the cycle, between 1300 and 1700 h.

2.5. Statistical analysis

The data were analyzed by two-way ANOVA, with the variable *Trial 1 length* as one factor and either the variable *trial* or *drug* as the second. Two-way ANOVA was followed by Duncan's test for multiple comparisons when necessary. Probability values less than 5% were considered significant. All statistical analysis was performed using the Statistica software package.

3. Results

Fig. 1 shows the %Open arm entries (Panel A) and %Open arm time (Panel B) exhibited by undrugged rats during 1, 2 and 5 min of Trial 1 exposure and retested (24 h later) for 5 min in the EPM. Two-way ANOVA failed to reveal a difference between groups relative to both %Open arm entries [$F(2,88) = 0.20$, P=.8124] and %Open arm time [$F(2,88) = 1.32$, P=.2713], either in Trial 1 or in Trial 2. However, two-way ANOVA showed a significant difference within groups in both %Open arm entries [$F(1,88) = 20.08$,

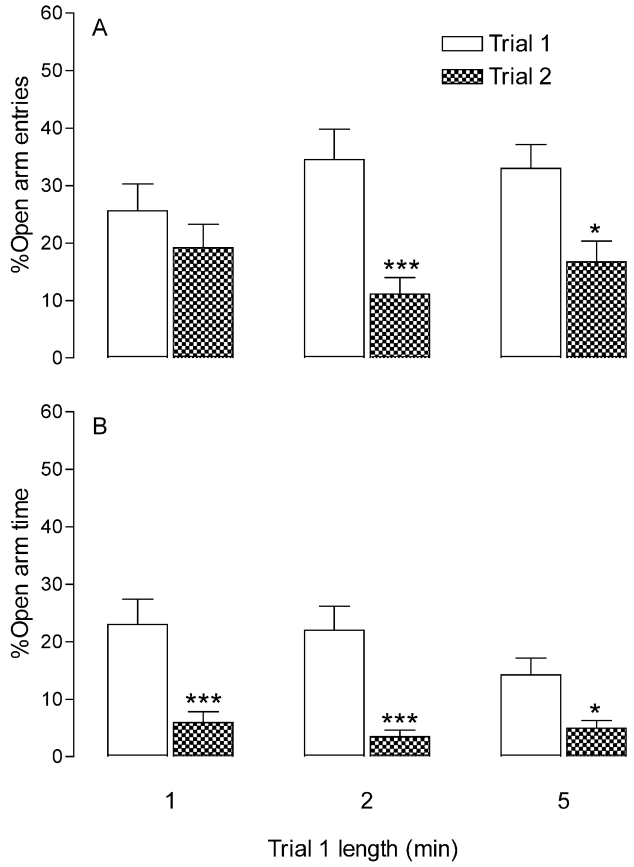

Fig. 1. Percentage of entries (Panel A) and time spent in the open arms (Panel B) during Trial 1 and Trial 2 in the EPM. Undrugged rats were previously exposed to Trial 1 with different lengths (1, 2 or 5 min) and subsequently exposed to a 5-min Trial 2. Data are expressed as the mean ± S.E.M. * $P < .05$ and * * * $P < .001$ relative to respective Trial 1 (two-way ANOVA followed by Duncan's test for multiple comparisons).

$P < .0001$] and %Open arm time [$F(1,88) = 37.78$, $P < .0001$]; Duncan's test for multiple comparisons revealed that both variables were reduced during Trial 2 if rats were previously exposed to the EPM for 2 ($P < .0001$) or 5 min ($P < .05$). Rats submitted to a 1-min Trial 1 displayed reduced %Open arm time ($P < .0001$), but not %Open arm entries ($P = .2847$), during Trial 2 relative to Trial 1. There was no interaction between the independent variables *Trial 1 length* and *trials*, neither in the %Open arm entries [$F(2,88) = 2.09$, $P = .1288$] nor in the %Open arm time [$F(2,88) = 1.41$, $P = .2493$].

Table 1 shows the number of entries into the open and enclosed arms, as well as the total number of arm entries in rats submitted to different Trial 1 lengths and retested for 5 min in the EPM 24 h later; ANOVA revealed a significant difference between groups relative to open [$F(2,88) = 3.27$, $P < .05$], enclosed [$F(2,88) = 4.83$, $P < .05$] and total arm entries [$F(2,88) = 4.78$, $P < .05$]. Duncan's test indicated that rats submitted to 5-min Trial 1 length displayed increased open ($P < .01$), enclosed ($P < .01$) and total arm entries ($P < .01$) relative to the group exploring the maze for 1 min during Trial 1. There was no difference between groups

Table 1
Number of entries into the open, enclosed and total number of arm entries in undrugged rats submitted to two consecutive trials in the EPM

Arm entries	Trial 1 length (min)	Trial 1	Trial 2
Open	1	1.26 ± 0.27	1.60 ± 0.44
	2	2.35 ± 0.36	1.23 ± 0.38
	5	3.53 ± 0.69 * *	1.66 ± 0.52 ##
Enclosed	1	3.06 ± 0.26	4.60 ± 0.60
	2	4.17 ± 0.38	5.70 ± 0.85
	5	6.20 ± 0.72 * *	5.80 ± 0.98
Total	1	4.33 ± 0.43	6.20 ± 0.98
	2	6.58 ± 0.52	6.94 ± 1.17
	5	9.73 ± 1.23 * *	7.33 ± 1.49

During Trial 1, rats were exposed to the EPM for 1, 2 or 5 min. Each group was retested for 5 min during Trial 2. The data are represented as the mean ± S.E.M.

** $P < .01$ relative to the group submitted to 1-min Trial 1 length.

$P < .01$ relative to respective Trial 1 (two-way ANOVA followed by Duncan's test for multiple comparisons).

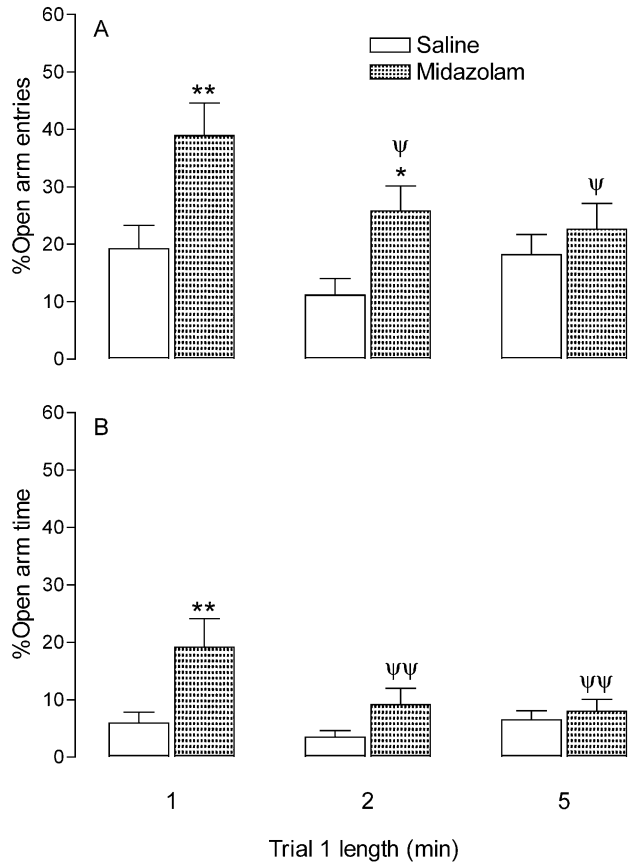

Fig. 2. Anxiolysis induced by MDZ during Trial 2 in the EPM. Rats (drug-free) were previously exposed to Trial 1 with different lengths (1, 2 or 5 min) and were subsequently retested for 5 min during Trial 2, 30 min after either saline (0.9%) or MDZ (1.5 mg kg^{-1}) intraperitoneal administration. Data are represented as the mean ± S.E.M. * $P < .05$ and * * $P < .01$ relative to respective group treated with saline. $^{\psi}P < .05$ and $^{\psi\psi}P < .001$ relative to the group previously exposed to 1-min trial length and treated with MDZ 30 min prior to Trial 2 (two-way ANOVA followed by Duncan's test for multiple comparisons).

in Trial 2, neither in the open and enclosed arm entries, nor in the total arm entries. ANOVA also revealed a significant difference within groups in the open [$F(1,88)=5.57$, $P<.05$], but not in the enclosed [$F(1,88)=2.53$, $P=.1151$] and total arm entries [$F(1,88)=0.005$, $P=.9435$]. Duncan's test indicated that rats submitted to a 5-min Trial 1 length exhibited lower open arm entries during Trial 2, relative to Trial 1 ($P<.01$).

Fig. 2 shows the %Open arm entries (Panel A) and %Open arm time (Panel B) exhibited by rats receiving either saline or MDZ prior to Trial 2; rats were undrugged during 1, 2 and 5 min of Trial 1 exposure. Two-way ANOVA revealed a significant difference between groups relative to both %Open arm entries [$F(2,80)=3.38$, $P<.05$] and %Open arm time [$F(2,80)=3.22$, $P<.05$], during EPM Trial 2. There was a significant difference within groups in both %Open arm entries [$F(1,80)=13.10$, $P<.0001$] and %Open arm time [$F(1,80)=9.67$, $P<.001$]; there was no interaction either in the %Open arm entries [$F(2,80)=1.79$, $P=.1720$] or in the %Open arm time [$F(2,80)=2.73$, $P=.070$]. Duncan's test revealed that in the group submitted to the 1-min Trial 1 length MDZ induced a full anxiolysis during Trial 2, since both %Open arm entries ($P<.01$) and %Open arm time ($P<.01$) were increased, relative to the group treated with saline; the MDZ-induced anxiolysis was not so clear in the group submitted to a 2-min Trial 1 length because only %Open arm entries was increased. MDZ was unable to induce anxiolysis in the group submitted to the 5-min Trial 1 length because neither %Open arm entries nor %Open arm time was changed by prior drug administration. The treatment with MDZ induced higher %Open arm entries ($P<.05$) and %Open arm time ($P<.01$) in the 1-min Trial 1 length group, relative to the 2- and 5-min Trial 1 length.

Table 2 shows the number of entries into the open, enclosed arms and the total number of arm entries in rats pre-exposed for 1, 2 or 5 min during Trial 1 and subsequently treated with either saline or MDZ, 30 min before Trial 2. ANOVA revealed a significant difference within [$F(1,80)=8.15$, $P<.01$], but not between groups [$F(2,80)=2.14$, $P=.1233$], relative to the number of entries into the open arm; Duncan's test indicated that in the group submitted to the 1-min Trial 1 length, the treatment with MDZ increased the number of entries into the open arms during Trial 2, relative to the group treated with saline ($P<.01$). ANOVA failed to reveal a significant difference either between or within groups relative to the enclosed [$F(2,80)=0.6893$, $P=.5048$ and $F(1,80)=0.2261$, $P=.6356$, respectively] and total arm entries [$F(2,80)=0.07$, $P=.9280$ and $F(1,80)=2.69$, $P=.1045$, respectively]. There was no interaction either in the number of entries into the open [$F(2,80)=2.51$, $P=.0874$], enclosed [$F(2,80)=0.27$, $P=.7590$], or in the total number of arm entries [$F(2,80)=1.17$, $P=.3154$].

Table 2
Number of entries into the open, enclosed and total number of arm entries exhibited by rats during Trial 2 in the EPM

Arm entries	Trial 1 length (min)	Saline	MDZ
Open	1	1.60 ± 0.44	4.50 ± 1.02$^{\#\#}$
	2	1.23 ± 0.38	2.50 ± 0.85
	5	1.66 ± 0.52	2.13 ± 0.49
Enclosed	1	4.60 ± 0.61	5.64 ± 0.72
	2	5.70 ± 0.85	5.87 ± 1.39
	5	5.80 ± 0.98	6.00 ± 0.93
Total	1	6.20 ± 0.98	10.14 ± 1.39
	2	6.94 ± 1.17	8.37 ± 2.16
	5	7.33 ± 1.49	8.13 ± 1.29

Animals were undrugged during different Trial 1 lengths and received either saline or MDZ 30 min prior to Trial 2. The data are represented as the mean ± S.E.M.

$^{\#\#}$ $P<.01$ relative to the respective group treated with saline (two-way ANOVA followed by Duncan's test for multiple comparisons).

4. Discussion

During the plus-maze exploration, the animal engages in exploratory behavior in a novel environment endowed with protected and unprotected spaces represented by the enclosed and open arms of the maze, respectively; the exposure of rats in either open or enclosed arms is a stressful experience since it induces increased corticosterone release relative to home-cage control rats; however, the open arms experience induces a significant increase in corticosterone blood level, relative to rats exposed to enclosed arms; in addition, rats exhibit a higher level of fear-related behavior in the open, relative to the enclosed arms (Pellow et al., 1985); thus, the open arms represent an area endowed with higher emotional meaning within the exploratory field of the animals in the EPM. The presence of defensive behaviors, such as the risk assessment towards the open while in the enclosed arm, indicates that a continuous gathering of information is occurring, which enables the animal to cope with the uncertainties/conflicts regarding the environment (Bertoglio and Carobrez, 2000).

During a first EPM experience, rats (Rosa et al., 2000) and mice (Holmes and Rodgers, 1998; Rodgers et al., 1996) exhibit an exploratory behavior characterized by progressive open arm avoidance and enclosed arms preference, indicating that the behavioral profile by the end is distinct from that displayed at the beginning of the test. The increased open arms avoidance has been attributed to the capacity of these arms to elicit fear-related behavior (Pellow et al., 1985) due to the rat's inability to engage in thigmotaxic behavior in open spaces (Treit et al., 1993; Cardenas et al., 2001). Since avoidance behavior represents an aversively motivated response, the presence of experience-dependent changes in the exploratory behavior over time, such as the open arm avoidance, denotes that fear-motivated learning is occurring during the first EPM exploration. In addition, memory processes appear to be facilitated during EPM exploration due to the release of endogenous inverse agonists, which induce desensitization of BZ receptors (Gonzales and File, 1997).

In the EPM, emotional memory can be evaluated through the Trial 1/Trial 2 paradigm, with the animals being re-exposed to the maze for 5 min, usually 24 h after Trial 1. The animal displays increased open arm avoidance in Trial 2, relative to Trial 1 (Espejo, 1997; Rodgers et al., 1996; Treit et al., 1993), indicating that prior EPM experience significantly increases the level of fear in a subsequent exposure. The present study showed that even though all experimental groups displayed increased fear during Trial 2, animals with 5 min to explore the maze exhibited higher open arm avoidance relative to Trial 1, while in animals with less time for exploration (1-min Trial 1 length group), the open arm avoidance seems to be less clear, suggesting that the emotional learning may be impaired by reduction in the time available for exploration during Trial 1. There was no locomotor habituation since the number of entries into the enclosed arms remained unchanged over both trials; in addition, it has been shown that no habituation occurs in the corticosterone response during Trial 2, since its release remains the same as that displayed during Trial 1 (File et al., 1994). Thus, increased fear, but not habituation, characterizes the animal's exploration during Trial 2 in the EPM.

Even though it had been proposed that the open arm experience is determinant in the avoidance learning process (File et al., 1998), rats previously exposed to either the open or the enclosed arms do not exhibit increased open arm avoidance when exposed to the EPM, displaying the same level of fear as maze-naive rats (Bertoglio and Carobrez, 2000); it appears that the exploration of an environment endowed with areas with different grades of emotional meaning is the crucial factor for fear-motivated learning to occur (Bertoglio and Carobrez, 2000). The present study showed that the time to cope with these distinct areas is also a critical variable for the establishment of the fear-motivated learning. Thus, reducing the available time to explore the EPM could result in less time to acquire information about the environment during Trial 1 and, consequently, impair the emotional learning during Trial 1; this assumption is in accordance with Rodgers et al. (1996) showing the importance of the initial minute in the acquisition of the avoidance learning.

The exposure of rats in the EPM induces increased Fos-like immunoreactivity in the amygdala (Duncan et al., 1996), a structure that appears to have a pivotal role in learned fear (Fendt and Fanselow, 1999) by modulating the emotional memory storage (Cahill and McGaugh, 1998). It has been shown that the increased open arm avoidance during EPM Trial 2 may be impaired by reversible bilateral lesions of the basolateral nucleus of the amygdala with lidocaine immediately after Trial 1 (File et al., 1998), suggesting that this region is important in the consolidation of the information acquired during the EPM Trial 1 and which underlies the increased open arm avoidance during Trial 2.

As a rule, when maze exploration occurs under a low level of fear, i.e. after anxiolytic-like drug administration, the animal displays a selective increase in the open, but not in the enclosed arm exploration (Pellow et al., 1985). However, it has been shown that BZs induce anxiolysis only in Trial 1, being unable to change the open arm avoidance in Trial 2 (File, 1990, 1993; File and Zangrossi, 1993). In the present study, the usual lack of the MDZ-induced anxiolysis in Trial 2 was observed only in the animals with more time available to explore the maze, while MDZ was able to induce a full anxiolytic effect in rats pre-exposed for 1 min in the EPM. Thus, the fear-motivated learning underlying the lack of MDZ-induced anxiolysis in Trial 2 is also dependent on the time available to explore the EPM during Trial 1. In this sense, the basolateral nucleus of the amygdala (File et al., 1998) and the dorsomedial hypothalamus (File et al., 1999) appear to be important for the consolidation of the information acquired during the EPM Trial 1 and retrieval of this information in Trial 2, respectively. In addition, it is possible that such emotional learning may be manifested even during Trial 1, since the anxiolytic effect induced by MDZ was impaired by the end of Trial 1 (Rosa et al., 2000).

It has been suggested that the absence of anxiolysis during Trial 2 may be due to acquisition of a phobic-like response to the open arms against which the BZs are not clinically effective (File, 1993); it has also been proposed that this phobic-like response could rapidly be extinguished, since it is not expressed if the animals are exposed for a more longer time to the phobic situation (File et al., 1993). In contrast, it has also been suggested that the recognition of the safety areas of the maze (Frussa-Filho and Ribeiro, 2002) or even the prior experience in the whole apparatus (Bertoglio and Carobrez, 2002) may be the crucial factor for the lack of anxiolysis induced by chlordiazepoxide during Trial 2. Thus, the basic requirements for fear-motivated learning in the EPM remain to be further determined.

The hypothesis of a qualitative change in the kind of fear parallels with clinical data, since anxiety is a heterogeneous psychiatric disorder with many forms of clinical manifestation, such as the generalized anxiety disorder, panic and phobia (American Psychiatry Association, 1994); thus, it is possible that emotional learning may underlie the qualitative shift of fear, with subsequent lack of BDZ-induced anxiolysis during Trial 2.

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